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THE EFFECT OF CHOLESTEROL ON PHAGOCYTOSIS *

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Cholesterol appears to be not only an essential constituent of the living cell and to play an important part in the construction of the organs, but also to be a factor in some of the reactions of immunity. In this latter capacity it is, to all appearances, the opposite to lecithin.

Cholesterol prevents the hemolytic action of such toxins as saponin, agaricin, tetanolysin, and vibriolysin. Windaus' method of separating cholesterol from its esters and other combinations is based on its property of forming compounds with toxins, and in particular with digitonin. The neutralizing action on toxins for erythrocytes is attributed to definite atoms in the molecule, according to Hausmann,[1] Abderhalden and LeCount,[2] and Walbum,[3] chiefly to the hydroxyl group. Schreiber and Lenard[4] tested the comparative antihemolytic action of cholesterol and oxycholesterol and observed that the latter prevents hemolysis only in higher concentration and in this respect is greatly inferior to cholesterol. This behavior changes as soon as oxycholesterol is added to cholesterol; the mixture has a stronger action than cholesterol alone in the same amount. Even the addition of a small quantity of oxycholesterol has this effect. The authors believe that this is due to the fact that the addition of oxycholesterol to cholesterol gives a better emulsion of the latter.

The relation of cholesterol to the reaction in the Wassermann test has been discussed by several writers. Pighini[5] found cholesterol in the spinal fluid in cases of dementia praecox and in epilepsy; the serum of these patients also contained abnormal amounts of cholesterol. According to him, this substance may be the chief active constituent in the Wassermann reaction. Walbum[3] believes that the action of cholesterol as antigen in this reaction is due to the free hydroxyl group. Gaucher, Paris, and Desmoulière[6] found an excess of cholesterol in the blood of old syphilitics to be the rule. Walker and Swift state that the antigen value of the extracts for the Wassermann reaction can be increased by the addition of cholesterol. According to Bürger and Beumer,[7] however, the outcome of the reaction is not dependent on the presence of simply more or less of cholesterol or lecithin.

Walbum,[3] Stuber,[8] and Arkin[9] studied the effect of cholesterol on phagocytosis. The experiments by Arkin with reference to this ques-

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1. Beitr. z. chem. Physiol. u. Path., 1905, 6, p. 567.
2. Ztschr. f. exper. Path. u. Ther., 1905, 2, p. 199.
3. Ztschr. f. Immunitsf., 1910, 7, p. 544.
4. Bioch. Ztschr., 1913, 54, p. 291.
5. Riforma medica, 1909, 25, p. 20.
6. Bull. acad. de Méd., 1912, 78, p. 55.
7. Berl. klin. Wchnschr., 1913, 3, p. 112.
8. Bioch. Ztschr., 1913, 51, p. 211; and 1913, 53, p. 493.
9. Jour. of Infect. Dis., 1913, 13, p. 408.

tion are but one in a series of tests of various pharmacological drugs for their action on phagocytosis, and were limited to observations in vitro. Walbum made his study in vitro and in vivo. In the former, cholesterol and a number of its derivatives were tested; of these, cholesterol itself and the benzoate and proprionate of cholesterol gave the highest figures. The experiments in vivo were carried out on three rabbits which received intravenous injections of cholesterol in doses of from 0.002 gm. to 0.0002 gm. per kilo of rabbit weight. The blood was taken before and several times after the injection and the serum examined for its opsonic strength with the colon bacillus. Stuber also studied the question by experiments both in vitro and in vivo. In the latter, cats were injected with doses of 30, 25, and 20 c.c. of a 0.5-1 percent suspension of cholesterol. The organism used was the thrush fungus. In both sets of experiments, the action of lecithin was also tested, chiefly with reference to its effect on the action of cholesterol.

The results have not been uniform. In Walbum's tests in vitro the figures of the absolute phagocytosis with cholesterol and its derivatives are all above normal phagocytosis, and those of cholesterol and the benzoate and proprionate are three times as much. From his experiments in vivo the author reports that phagocytosis was doubled and that this action seemed to remain unchanged for a long time. He concluded that since the cholesterol action sets in at once, it might perhaps be of interest to test this method therapeutically. It seemed to him, however, that an increased amount of cholesterol did not alter the action; he states that his experiments were too limited for any definite conclusions. Stuber's results are in striking contrast. The experiments in vitro did not vary in results notably from those in vivo, which were carried out on cats; there was a decided fall in phagocytosis, amounting to from 40 to 70 percent. Lecithen neutralized the inhibiting action of cholesterol; when lecithin is heated for fifteen minutes at 70 C. the neutralizing action is abolished. The action of lecithin on cholesterol was of short duration.

Arkin also obtained a diminished phagocytosis in vitro.

In view of the marked difference in the results of Walbum and of Stuber, it seemed worth while to make a further study of cholesterol with reference to phagocytosis. The object was to determine the opsonic power of the serum of guinea-pigs injected intraperitoneally with increasing doses of cholesterol and that of an animal immunized with streptococci and simultaneously injected with cholesterol in the same doses as used with the non-immunized animal.

The experiments were continued for twenty days. Thirteen injections were made, nine with an average interval of thirty-six hours, the last four in daily succession. Merck's pure cholesterol was used in a colloidal suspension prepared by the method of Porges and Naubauer,[10] which was employed also by Walbum and Stuber. A 1 percent suspension in doses from 0.1 to 0.4 c.c. was given. Suspensions of killed streptococci in doses from 100,000,000 to 400,000,000 were given in nine successive injections, which were followed by four injections with living streptococci in doses of from 100,000,000 to 300,000,000. A normal guinea-pig and one injected with strepto-

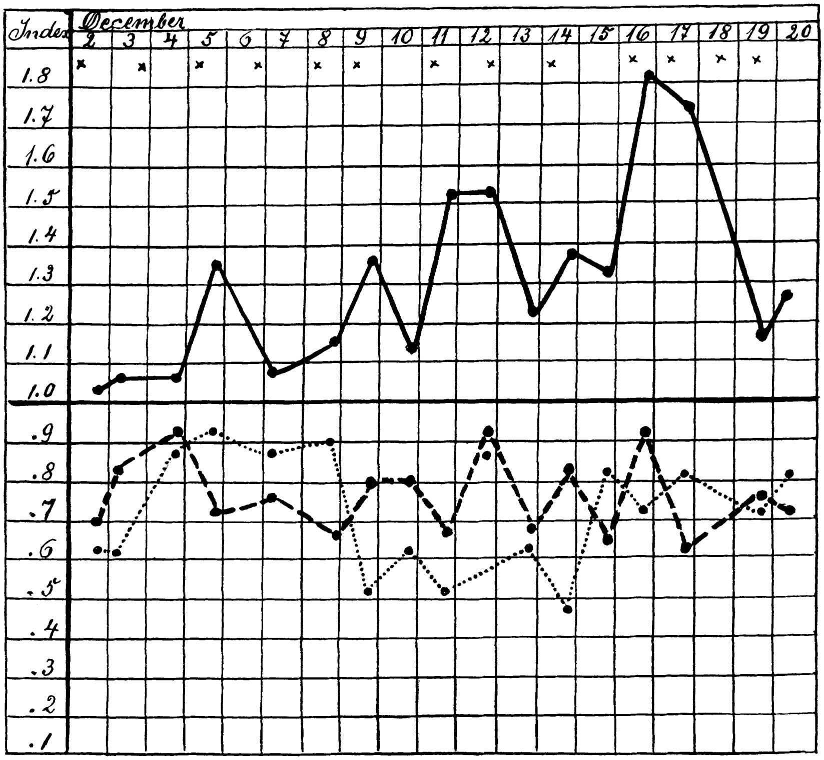

Chart 1.—Curves of opsonic index.

Heavy solid line = guinea-pig immunized against streptococci.
Dotted line = guinea-pig injected with cholesterol.
Heavy broken line = guinea-pig injected with streptococci and cholesterol.
The crosses give the time of the injections of cholesterol.

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[10] Biochem. Ztschr., 1907, 7, p. 152.

cocci served as control animals. Blood was taken from the animals once, and a few times twice, during the period between two injections; human blood furnished the white corpuscles; the organisms for phagocytosis were streptococci. The serum from each animal was mixed with an equal amount of washed human leukocytes and of a streptococci suspension and incubated for fifteen minutes at 37 C. Smears were stained with Leishman's stain. With a few exceptions, 100 cells were counted; the figures of the normal control animal were taken as the unit for the phagocytic ratio. The opsonic curves on Chart 1 illustrate the results obtained.

We see that by introducing small quantities of cholesterol into the systems of guinea-pigs, and thus keeping up a constant supply of this substance in the circulation, there is, in the action of the serum of such animals on washed normal human leukocytes, a reduction of phagocytosis as compared with the action of normal serum of guinea-pigs. This diminution is from 20 to 30 percent. The simultaneous injection of immunizing doses of killed streptococci with the cholesterol results in an opsonic curve which is almost identical with that of serum containing only cholesterol. In these experiments only the serum was tested. These results from the injections of cholesterol are in marked contrast to those reported by Walbum and agree to a large extent with those of Stuber, tho our figures of the reduced index are not as low as his. Since his methods deviate from mine and were chosen with a different object in view, it seemed of interest to study the question with the same methods.

Stuber wanted to study the effect of cholesterol on the leukocytes; one-half hour after the intravenous injection of cholesterol into cats, in quantities which are large in comparison to those used by Walbum and ourselves, 0.03 c.c. of blood was mixed with 0.03 c.c. of a suspension of thrush fungus and 0.1 c.c. of a saturated solution of ovalbumen, (or 0.1 c.c. of artificial serum or 0.1 c.c. of the serum of the same species of animal). This mixture was incubated for one hour at 37 C. and smears were made of the leukocytes which were separated from the mixture by a short centrifugation; 200 leukocytes were counted. Stuber observed that if the leukocytes were washed their phagocytic function was affected; he abstained from this customary method. He obtained a decrease in phagocytosis by 40 to 70 and even 90 percent, and concluded that the action of cholesterol is on the leukocytes themselves, their vital functions being paralyzed. He is not inclined to consider the opsonins of the serum to have the significance which Wright attaches to them. "The conception of opsonins," he writes, "is only the relatively comprehensible expression of possibilities of biological reactions between lipoids of the cell and those of the serum, so that we may conceive of a certain lipoid tonus, to which the cell, bathed in serum, is subjected, and whose visible biologic effect on the leukocytes is manifested by phagocytosis." Stuber found the injurious effect of cholesterol on the vital functions of the leukocytes to be of long duration, as on the sixth day after the injection there was still a reduction of phagocytosis of 50 percent. The accuracy of Stuber's methods seems reliable, but those to whom opsonins have a practical meaning may question whether his interpretation of his results is the only one possible; the objection might be raised that in his tests of phagocytic power the possibility that the cholesterol acted on the opsonins cannot be excluded.

In the study of this question the following method was used. The opsonic index of three rabbits was tested several times before the injections were made; the corpuscles of each animal were used in succession and no variations in the phagocytic ratio were found beyond those within the limit of error in the technic. Spontaneous phagocytosis was tested also. The index obtained the day before the injection is a fair average of these counts: Rabbit A, 1,320 gm., 1.28; Rabbit B, 1,390 gm., 1.30; Rabbit C, 1,420 gm., 1.20; while spontaneous phagocytosis was 0.28.

Rabbit A was used as control animal No. 1; Rabbit B was injected intraperitoneally with 0.6 c.c. of a freshly prepared 0.5 percent suspension of cholesterol; Rabbit C, was control animal No. 2 and received a similar dose as Rabbit B without the cholesterol, namely, distilled water to which acetone had been added and evaporated. One hour, forty-eight, and seventy-two hours after the injection, the blood was taken from each animal for the serum and for the leukocytes. Coagulation was prevented with citrate solution; the bacterium employed was the staphylococcus, and preparations were stained with carbolthionin.

The results show a definite and constant, tho not very marked, reduction of phagocytosis with normal serum on leukocytes acted on by cholesterol and with serum containing cholesterol on normal leuko-

TABLE 1

EFFECT OF A SMALL DOSE OF CHOLESTEROL ON THE LEUKOCYTES

Time of Taking Blood for Serum and Leukocytes	Leukocytes	Serum	(Phagocytosis (Absolute Number of Bacteria per Cell)	Ratio
1 hour after the injection	Rabbit A, Control 1	Rabbit A	2.02	1.00
		Rabbit B	1.48	0.72
		Rabbit C	2.05	1.02
	Rabbit B injected with 0.6 c.c. 0.5 percent suspension of cholesterol	Rabbit A	1.72	1.00
		Rabbit B	0.01	0.35
		Rabbit C	1.76	1.02
	Rabbit C, Control 2; injected with 0.6 c.c. distilled water.	Rabbit A	1.93	1.00
		Rabbit B	1.44	0.74
		Rabbit C	2.00	1.03
72 hours after the injection	Rabbit A, Control 1	Rabbit A	1.54	1.00
		Rabbit B	1.22	0.79
		Rabbit C	1.50	0.98
	Rabbit B, injected with cholesterol	Rabbit A	1.28	1.00
		Rabbit B	0.88	0.69
		Rabbit C	1.20	0.99
	Rabbit C, Control 2, injected with distilled water	Rabbit A	1.56	1.00
		Rabbit B	1.28	0.82
		Rabbit C	1.54	0.99

TABLE 2

Comparison of the Action of Serum and of the Leukocytes in Phagocytosis after the Injection of a Small Dose of Cholesterol

Time of Taking Blood	Normal Serum on Normal Leukocytes	Normal Serum on Leukocytes of Animal Injected with Cholesterol	Serum of Animal Injected with Cholesterol on Normal Leukocytes	Serum of Animal Injected with Cholesterol on Its Own Leukocytes
1 hour after the injection	2.02	1.72	1.48	0.61
	2.05			
	1.93	1.76	1.44	
	2.00			
Ratio	1.00	0.86	0.73	0.35
72 hours after the injection	1.54	1.28	1.22	0.88
	1.50			
	1.56	1.20	1.28	
	1.54			
Ratio	1.00	0.80	0.81	0.57

TABLE 3

Effect of a Large Dose of Cholesterol on the Leukocytes

Time of Taking Blood for Serum and Leukocytes	Leukocytes	Serum	Phagocytosis (Absolute Number of Bacteria per Cell)	Ratio
1 hour after injection	Rabbit A, Control 1	Rabbit A	3.46	1.00
		Rabbit B	2.26	0.67
		Rabbit C	3.34	0.96
24 hours after injection	Rabbit A, Control 1	Rabbit A	3.60	1.00
		Rabbit B	2.36	0.65
		Rabbit C	3.48	0.97
	Rabbit B, injected with 7 c.c. 0.5 percent suspension of cholesterol	Rabbit A	2.36	1.00
		Rabbit B	1.35	0.57
		Rabbit C	2.26	0.96

TABLE 4

Comparison of the Action of Serum and of the Leukocytes in Phagocytosis after the Injection of a Large Dose of Cholesterol

Time of Taking Blood	Normal Serum and Normal Leukocytes	Normal Serum and Corpuscles of Animal Injected with Cholesterol	Serum of Animal Injected with Cholesterol on Normal Leukocytes	Serum of Animal Injected with Cholesterol on Its Own Leukocytes
1 hour after injection	3.40	....	2.26	....
24 hours after injection	3.60	2.36	2.36	1.35
	3.48	2.26		
Ratio	1.00	0.65	0.67	0.38

cytes. The figure obtained with the serum and leukocytes of the animal injected with cholesterol shows an absolute reduction in phagocytosis of 65 percent after one hour, and of 43 percent seventy-two hours after the injection of a single small dose of cholesterol.

A large dose was then injected corresponding in quantity and concentration to some of those employed by Stuber, viz., 7 c.c. of a 0.5 percent suspension of cholesterol; 7 c.c. of distilled water plus acetone, the latter having been evaporated as in the preparation of the cholesterol suspension, were injected into control animal No. 2.

The relations of the index are approximately the same as in the experiments with a small dose, but the reduction is greater, which effect is attributed to the larger amount of cholesterol.

It is evident from these results that the action of cholesterol is not on the opsonin alone, if at all. If it were so and cholesterol would reduce the opsonic power of the serum simply by combining with the opsonin, as Arkin suggests, we would not have the constant effect of a reduced phagocytosis with normal serum on the washed leukocytes of cholesterol Rabbit B. This would rather indicate that the leukocytes have been altered by cholesterol so as to lower their phagocytic powers. Serum containing cholesterol produced the same degree of reduction in the phagocytic power of normal leukocytes within the short period of incubation as normal serum with leukocytes altered by cholesterol. This reduction is 20 to 30 percent after the small dose and 35 percent after a larger dose. The opsonic index of the serum containing cholesterol with leukocytes injured by, or acted on, by this substance is enormously diminished; the absolute reduction is 65 percent one hour after the injection of a small dose; 43 percent seventy-two hours after the same injection; and 62 percent twenty-four hours after the larger dose. These figures closely approach those obtained by Stuber. That opsonin and serum apparently have little to do with the diminished phagocytic function of the leukocytes was demonstrated by Stuber's experiments, in which the quantity of serum was reduced to a minimum. The limited number of our experiments does not permit any conclusion to be drawn as to the method in which this occurs. Besides the direct detrimental effect on the leukocytes there may be interaction with the opsonin whereby the leukocytes are injured, and there may be some effect on the bacteria; but it is not unlikely that the effect is directly and entirely on the leukocytes, as Stuber believes. This behavior of the leukocytes toward phagocytosis under the influence of cholesterol runs perhaps parallel with antihemolytic action.

Mayerstein and Allenbach[11] state that leukocytes are generally capable of hindering hemolysis. This action, they claim, depends on their absorption of hemolytic agents, whereby they are deviated from the erythrocytes. The absorption takes place not only by intact leukocytes, but also by broken-up cells and is perhaps chiefly dependent on their content of lipoid substances. "The paralyzing effect of cholesterol on the vital functions of the leukocytes," Stuber writes, "recalls the action of the iodin ion." He showed that by substitution of the hydroxyl group of the cholesterol molecule, such as occurs in cholesterylbenzoate, cholesterol loses its inhibiting power, and the neutralizing effect of lecithin on the action of cholesterol is, according to him, due to the anchoring of the hydroxyl group of lecithin. This important group of the cholesterol molecule is therefore a chief factor in its action on the vital functions of the leukocytes. The recovery of the cells from the injury takes place after a relatively long period of time, as Stuber found phagocytosis still reduced by 50 percent on the sixth day.

Walbum obtained results which differed from those of Stuber and us, and his conclusions naturally are different. He found phagocytosis greatly increased by cholesterol, and according to him, this action is apparently not dependent on the free hydroxyl group, but rather a specific one and not an ordinary colloid phenomenon. It is not altogether impossible and his results are, in part, due to the concentration of the suspension. Dilutions with distilled water twenty times greater than those employed by Stuber and ourselves were used for the injections as well as the experiments in vitro. No serum nor salt solution was added to these high dilutions with distilled water. It would seem from a test in vitro made by Arkin that cholesterol alone has a stimulating effect on phagocytosis; without serum and without salt solution the phagocytic index of the leukocytes with cholesterol was 1.54, i. e., more than half of that of serum alone, which was 2.74. That of cholesterol with salt solution was 1.94; with serum 1.18.

In order to determine the effect of cholesterol on immunization, two weeks after the last injection our animals were used for another experiment. A blood test, made at this time, showed complete absence of cholesterol action both on leukocytes and on the serum. One cubic centimeter of a suspension of killed streptococci, containing 100,000,000 organisms, was injected into Rabbit A, and 7 c.c. of a 2 percent suspension of cholesterol were injected into Rabbit B. The same strain of streptococci, a rather virulent one, was used for phagocytosis. The serum of the rabbits was tested before and one hour after the injection; twenty-four hours following this a test was made again and the same injections in reversed order were made; 7 c.c. of a 2 percent suspension of cholesterol were injected into Rabbit A, which had received the vaccine, and 100,000,000 killed streptococci into Rabbit B, which had received cholesterol. One hour after these injections blood was taken for examination with the leukocytes of Rabbit C.

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[11] Biochem. Ztschr., 1913, 58, p. 93.

The figures of phagocytosis in Rabbit A, injected with killed streptococci, with serum taken before and one hour and twenty-four hours after the injection, indicate a negative phase one hour, and a decided rise twenty-four hours after the vaccine injection. A large dose of a concentrated suspension of cholesterol is followed by an enormous decrease in phagocytosis one hour after the injection, which amounts to 69 percent. Rabbit B shows a fall of phagocytosis by 64 percent one hour after the injection of cholesterol, which after twenty-four hours is only 24 percent. One hour after the inoculation of killed streptococci. the ratio of phagocytosis is reduced from 0.76 to 0.36, that is, by 53 percent.

TABLE 5

THE EFFECT OF CHOLESTEROL ON PHAGOCYTOSIS IN RELATION TO IMMUNIZATION BY KILLED STREPTOCOCCI

Animal	Opsonic Index before Injection	Dose and Order of Injection	Opsonic Index 1 Hour after Injection		Opsonic Index 24 Hours after Injection		Dose and Order of Injection	Opsonic Index 1 Hour after Injection	
			Absolute	Ratio	Absolute	Ratio		Absolute	Ratio
Rabbit A	0.52	1 c.c. = 100 million killed strep.	0.48	0.86	0.88	1.16	7 c.c. 2 percent suspension cholesterol	0.28	0.36
Rabbit B	0.52	7 c.c. 2 percent suspension cholesterol	0.20	0.36	0.58	0.76	1 c.c. = 100 million killed streptococci	0.28	0.36
Rabbit C	0.54	Control	0.56	1.00	0.76	1.00	Control	0.78	1.00

The immediate effect of immunization on the cholesterol action and of cholesterol on immunization is striking, both resulting in a marked depression of phagocytosis, and resembles the effect which was observed in the first series of experiments on guinea-pigs, when cholesterol and killed streptococci were injected simultaneously; the curve of the indices of this animal closely follows that of the animal injected with cholesterol alone. Outside of these pronounced results, this test does not furnish us with a clue to explain the reactions. Immunization by bacteria is supposed to increase the opsonins and we actually see a definite, tho not very marked, rise in the opsonic index twenty-four hours after the inoculation. The fall in the ratio by 69 percent one hour after the injection of a large dose of a concentrated suspension of cholesterol into the animal is slightly more than the one after an initial dose of cholesterol of the same quantity and concentration in Rabbit B, an effect, seemingly, of a complete neutralization of the influence of immunization on the opsonin. More striking is the result of a dose of killed streptococci in a rabbit which twenty-four hours previous to this has received a large dose of cholesterol; the opsonic index of 0.36 one hour after the injection had risen to 0.76. The vaccine injection is followed by a fall in phagocytosis by 53 percent. If we hold that immunization acts on the opsonin and we assume that in this case there was a marked negative phase, part of the great reduction is still to be attributed to other reactions.

In these tests of the relation of cholesterol to immunization, the condition and function of the leukocytes acted on by cholesterol and the antigenic substances are not taken into account. A consideration of these will be made later. The question of the relations between cholesterol and immunization seems to be of great interest. We are naturally led to ask: how does cholesterol, which is normally present in the blood, and in most of the organs of the human body and increased in a number of pathological conditions, act in infectious processes? Further investigations may give answer to this question that may have important bearing on therapy.

SUMMARY

It appears that cholesterol in colloidal suspension, injected into guinea-pigs and rabbits, has a depressing effect on phagocytosis.

This action is probably chiefly on the leukocytes; for the opsonic index of normal serum with the washed leukocytes of an animal injected with cholesterol is lower than that of the same serum with washed normal leukocytes, and the index of the serum containing cholesterol with the washed leukocytes of the same blood shows a reduction in phagocytosis more than twice as great as that of such serum with washed normal leukocytes.

The fall of the total phagocytic index, that is, of the serum of the animal injected with cholesterol acting on its own washed leukocytes, is 65 percent after one hour, and 43 percent seventy-two hours after one small dose, and 62 percent twenty-four hours after one large dose of cholesterol.

The chief difference in the results from injections of small and large amounts of cholesterol and of doses of higher and lower concentration seems to be, as far as the range of these experiments indicates, a more marked immediate effect from the larger dose and from that of higher concentration.

This inhibitory action of cholesterol is perhaps due to the presence of free cholesterol in the blood.

Simultaneous injections of immunizing doses of streptococci with cholesterol result in an opsonic curve which is almost identical with that of the serum of an animal injected only with cholesterol. This apparent neutralizing effect of cholesterol on the formation of antibodies is marked when cholesterol is injected twenty-four hours after the animal has received the immunizing dose.